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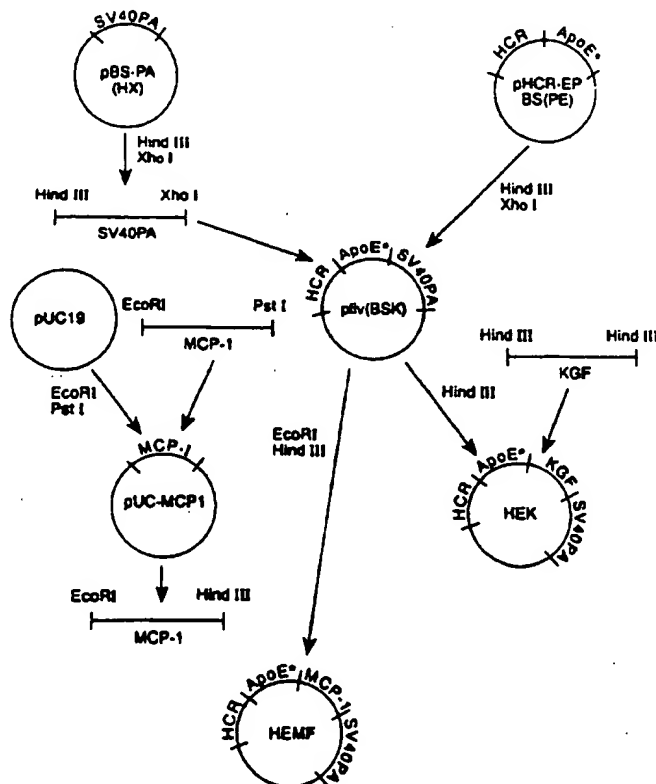# INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: MAMMAL WITH ENHANCED LIVER EXPRESSION OF A TRANSGENE

(57) Abstract

This invention provides a mammal with enhanced liver expression of a transgene. Also provided are: 1) a nucleic acid sequence useful in enhancing liver specific expression of a transgene, and 2) a vector containing this nucleic acid sequence.

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MAMMAL WITH ENHANCED LIVER EXPRESSION OF A TRANSGENE.

This application is a continuation-in-part of
U.S.S.N. 08/141,322 filed October 18, 1993.

5

BACKGROUND

FIELD OF THE INVENTION

10   This invention relates to the field of
recombinant DNA technology, especially to nucleic acid
sequences useful for constructing a transgenic mammal.
More specifically, the invention concerns expression of
a transgene in certain tissues or organs of a mammal.

15

DESCRIPTION OF RELATED ART

1. Tissue Specific Expression

20   Production of a transgenic mammal involves the
insertion of a nucleic acid sequence, often called a
transgene, which codes for a particular polypeptide,
into one or more chromosomes of the mammal. This is
typically accomplished by inserting the transgene into

25   the pronucleus of an isolated mammalian egg. The
transgene becomes incorporated into the DNA of the
developing embryo. This embryo is then implanted into a
surrogate host for the duration of gestation. The
offspring of the surrogate host are evaluated for the

30   presence of the transgene.
        Expression of the transgene, i.e., production
of the protein encoded by the transgene nucleic acid
sequence, may confer a new phenotype on the mammal.
Depending on the transgene(s) inserted into the animal

35   and the level of expression of the transgene in the
mammal, the mammal may become more or less susceptible

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to a particular disease or series of diseases. Such
transgenic mammals are valuable for *in vivo* screening
and testing of compounds that may be useful in treating
or preventing the disease(s), and/or for developing
5   methods useful in diagnosing the disease.

While methods for insertion of a novel gene
into a mammal have developed rapidly, several problems
with the application of this technology remain. One
such problem concerns limiting expression of the gene
10   primarily to a selected tissue or tissues where
expression is desired.

Enhanced and/or specific expression of a gene
in a select tissue or tissues of a mammal is complex.
Expression of a gene is typically regulated at least in
15   part by a non-coding nucleic acid sequence termed a
promoter. The promoter is often located near or
adjacent to the nucleic acid sequence encoding the
polypeptide to be expressed. Frequently, the activity
of a promoter is in turn regulated by other nucleic acid
20   sequences termed enhancers and suppressors (also known
as silencers). Enhancers increase the level of
expression of the gene while suppressors or silencers
decrease expression. The location of enhancers and
suppressors along a nucleic acid sequence with respect
25   to the promoter and coding sequence is quite varied for
different genes. Enhancers and suppressors may be
located near or adjacent to the promoter, i.e., within
about 1 kilobase (kb) along a strand of DNA (chromosome
or vector), or may be located at a much greater
30   distance, *e.g.*, up to 50 kb or more away from the
promoter on a chromosome and still exert an effect on
the activity of the promoter. Further, they may be
located upstream (*i.e.*, 5' to the promoter and coding
sequence), or downstream (3' to the promoter and coding
35   sequence). Such positioning for promoter activity is a
function of both the type of promoter and the type of

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enhancer or suppressor used. To further complicate the
regulation, enhancers and suppressors may exert their
effect on the promoter of more than one gene within a
chromosomal locus.

5   Several enhancers and suppressors have been
identified. For example, the level of expression of the
gene encoding transthyretin is affected by an enhancer
element located about 2 kb upstream from the promoter
(Yan et al., EMBO J., 9:869-AFM8 [1990]). Liver

10 specific expression of the albumin gene is regulated by
an enhancer located about 10 kb upstream of its promoter
(Hammer et al., Science, 235:53-58 [1987]). Tissue
specific regulation of the alpha-fetoprotein gene
involves three enhancer elements located 1 to 7 kb

15 upstream of the transcription start site of the gene
(Pinkert et al., Genes & Dev., 1:268-276 [1987]).

           Another enhancer is the hepatocyte-specific
control region, or "HCR". The human HCR is believed to
be about 774 base pairs (bp) in size or less (Simonet et

20 al., J. Biol. Chem., 268: 8221-8229 [1993]), but has
recently been reported to be at least somewhat active as
a 150 to 154 bp fragment (Breslow, Proc. Natl. Acad.
Sci. USA, 90:8314-8318 [1993]; Shacter et al., J. Lipid
Res., 34:1699-1707 [1993]). The HCR is located on

25 chromosome 19, about 18 kilobases (kb) downstream of the
apolipoprotein E (apoE) promoter/gene sequence, about 9
kilobases downstream of the apolipoprotein C-I (apoC-I)
promoter/gene sequence, and about 2 kilobases (kb)
upstream of the apolipoprotein C-I (apoC-I') pseudogene

30 sequence (Simonet et al., [1993], supra; Simonet et al.,
J. Biol. Chem., 266:8651-8654 [1991]; Simonet et al., J.
Biol. Chem., 265:10809-10812 [1990]; Taylor et al.,
Current Opinion in Lipidol., 2:73-80 [1991]). The HCR
appears to be important in expression of the genes ApoE

35 and ApoC-I in the liver; in its absence, these genes are

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not expressed at detectable levels in this tissue
(Simonet *et al.* [1993], *supra*).

The effect of the HCR on a heterologous
promoter has been evaluated in transgenic mice. The
5 apolipoprotein A-IV promoter and coding sequence were
ligated to a 1.7 kb nucleic acid sequence containing the
HCR. Transgenic mice containing this construct had high
levels of expression of apolipoprotein A-IV in the liver
(Simonet *et al.*, *supra*).

10

2. Interleukin-8

The interleukins are a group of naturally
occurring proteins that act as chemical mediators of the
15 differentiation processes for red and white blood cells.
One of the interleukins, IL-8 (also known as Neutrophil
Activating Peptide-1, or NAP-1), has been shown to be a
neutrophil chemoattractant with the ability to activate
neutrophils and stimulate the respiratory burst (Colditz
20 et *al.*, *J. Leukocyte Biol.*, 48:129-137 [1990]; Leonard
et *al.*, *J. Invest. Derm.*, 96:690-694 [1991]). IL-8 has
been termed a proinflammatory cytokine due to its
involvement in neutrophil recruitment to sites of acute
and chronic inflammation.
25 Zwahlen *et al.* (*Int. Rev. Exp. Path.*, 34B:22-
42 [1993]) describe some effects of IL-8 injected into
some rodents. When injected intradermally into rats,
IL-8 induced neutrophil infiltration at the site of
injection. Intravenous injection of IL-8 into rabbits
30 resulted in neutrophil sequestration in the lungs.
Vogels *et al.* (*Antimicrobial Agents and
Chemotherapy*, 37:276-280 [1993]) describe the effect of
administering IL-8 to mice either before or after
infection of the mice with three different pathogens.
35 Under certain conditions, administration of IL-8 was

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shown to have a detrimental effect on the survival of the mice.

Van Zee et al. (J. Immunol., 148:1746-1752 [1992]) describe administration of IL-8 to baboons. The animals developed neutropenia rapidly after IL-8 administration. This neutropenia is transient and is followed by a marked granulocytosis which persists for as long as IL-8 is present in the circulation.

Burrows et al. (Ann. NY Acad. Sci., 629:422-424 [1991]) show that guinea pigs injected with IL-8 had a higher level of T-lymphocyte and eosinophil accumulation in the lung than did control animals.

3. Keratinocyte Growth Factor

Keratinocyte growth factor (KGF) is a mitogen that has been identified as specific for epithelial cells, especially keratinocytes (Rubin et al., Proc. Natl. Acad. Sci. USA, 86:802-806 [1989]; Finch et al., Science, 245:752-755 [1990]; Marchese et al., J. Cell Physiol., 144:326-332 [1990]). KGF has shown potential for repair of epidermal tissues such as the skin, and epithelial tissues of the digestive tract. The DNA encoding KGF has been cloned and sequenced (PCT 90/08771, published August 9, 1990).

Guo et al. (EMBO J., 12:973-986 [1993]) have prepared a transgenic mouse containing a transgene constructed of the human keratin 14 promoter and the human keratinocyte growth factor gene. The mouse showed a number of phenotypic differences as compared with non-transgenics such as wrinkled skin and reduced hair follicle density.

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4. Monocyte Chemoattractant Protein

Monocyte chemoattractant protein (also known
as MCP-1) is a protein that is produced by activated
5   leukocytes in response to certain stimuli. The gene
encoding human MCP-1 has been cloned and sequenced
(Furutani et al., Biochem. Biophys. Res. Comm., 159:249-
255 [1989]; Yoshimura et al., Chemotactic Cytokines,
Westwood et al., eds. Plenum Press, NY [1991], pp.47-
10   56). MCP-1 serves to attract monocytes to the site of
its release, and is believed to be involved in the
cellular immune response and in acute tissue injury
(Leonard et al., Immunol. Today, 11:97-101 [1990]).
MCP-1 has been shown to be produced by some tumor cells
15   in vitro, and in human metastatic melanomas in vivo
(Graves et al., Am J. Pathol., 140:9-14 [1992]).

5. Human Afamin

20   Afamin ("AFM") is a novel protein recently
identified in human serum. AFM has a molecular weight
of about 87,000 daltons when run on SDS_PAGE, and shares
significant homology to members of the albumin family of
proteins including vitamin D binding protein (VDB),
25   alpha fetoprotein, and albumin. In addition, AFM has
the characteristic pattern of disulfide bonds observed
in this family. AFM cDNA has been stably transfected
into Chinese hamster ovary cells, and recombinant AFM
(rAFM) has been purified from the conditioned culture
30   medium of these cells. Both AFM and rAFM react with a
polyclonal antibody that was raised against a synthetic
peptide derived from the deduced amino acid sequence of
AFM.   There is a need in the art to provide in vivo
systems for evaluating the effects of one or more genes
35   on certain diseases.

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Accordingly, it is an object of this invention
to provide a mammal containing a nucleic acid construct
comprising a transgene, and expressing the transgene,
where the mammal may be used as an *in vivo* system to
5   analyze the course of a disease.

It is a further objective to provide a
transgene nucleic acid construct and an expression
vector that enhance tissue specific expression of a
transgene in liver tissue of a transgenic mammal.

10   Other such objects will readily be apparent to
one of ordinary skill in the art.

SUMMARY OF THE INVENTION

15   In one aspect, the present invention provides
a nucleic acid sequence comprising an HCR enhancer
operably linked to a promoter and a transgene. The
promoter may be selected from the group of promoters
consisting of: ApoA-I, ApoA-II, ApoA-III, ApoA-IV, ApoB-
20   48, ApoB-100, ApoC-I, ApoC-II, ApoC-III, ApoE, albumin,
alpha feto protein, PEPCK, transthyretin, SV40, CMV, and
TK. The transgene may be selected from the group
consisting of: interleukin 1, interleukin 2, interleukin
3, interleukin 4, interleukin 5, interleukin 6,
25   interleukin 7, interleukin 8, interleukin 9, interleukin
10, interleukin 11, interleukin 12, ENA-78, interferon-α,
interferon-β, interferon-γ, granulocyte-colony stimulating
factor, granulocyte-macrophage colony simulating factor,
macrophage colony stimulating factor, stem cell factor,
30   keratinocyte growth factor, MCPI,
AFM, and TNF, and fragments thereof.

In one other aspect, the invention provides a
non-human mammal and its progeny containing a nucleic
acid sequence comprising an HCR enhancer operably linked
35   to a promoter and a transgene.

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The invention further provides a non-human transgenic mammal containing nucleic acid sequence comprising an HCR enhancer, the human ApoE promoter, the human ApoE intron 1 linked at its 5' end to the human
5   ApoE exon 1 and at its 3' end to a portion of the human ApoE exon 2, and at least a portion of the coding sequence of the transgene human IL-8, the transgene KGF, or the transgene AFM.

10   BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleic acid sequence of the 774 base pair human HCR (SEQ ID NO: 1). This sequence was derived from the vector pCI-CI'PX#8,
15   deposited with the American Type Culture Collection (ATCC).

Figure 2A-C depict the transgene construct used to generate IL-8, KGF, and MCP-1 transgenic mice.
20   Vectors are labeled as referenced in the Examples. Selected restriction enzymes are shown. "ApoE*" refers to the ApoE promoter, first exon, first intron and a portion of the second intron; "SV40PA" refers to the SV40 polyA+ sequence, as described in the Examples.
25
Figure 3 depicts the level of IL-8 and circulating neutrophils in both control and transgenic mice. Figure 3A shows serum IL-8 levels. Figure 3B shows circulating neutrophil levels. NT represents non-
30   transgenic (control) mice. The numbers refer to individual lines of transgenic mice used in the analysis.

Figure 4A-C depicts a nucleic acid molecule
35   (cDNA) of approximately 2.3 kb encoding human AFM (SEQ ID NO:23). The translated amino acid sequence of AFM is

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also shown (SEQ ID NO:24). The amino acid positions are
numbered, with -21 through -1 being the signal peptide
sequence, and 1-578 being the mature protein sequence.

5

DETAILED DESCRIPTION OF THE INVENTION

<u>Definitions</u>   .

10   The term "operably linked" refers to the
arrangement of various nucleic acid molecule elements
relative to each such that the elements are functionally
connected and are able to interact with each other.
Such elements may include, without limitation, a
15 promoter, an enhancer, a polyadenylation sequence, one
or more introns and/or exons, and a coding sequence of a
gene of interest to be expressed (*i.e.*, the transgene).
The nucleic acid sequence elements, when properly
oriented or operably linked, act together to modulate
20 the activity of one another, and ultimately may affect
the level of expression of the transgene. By modulate
is meant increasing, decreasing, or maintaining the
level of activity of a particular element. The position
of each element relative to other elements may be
25 expressed in terms of the 5' terminus and the 3'
terminus of each element, and the distance between any
particular elements may be referenced by the number of
intervening nucleotides, or base pairs, between the
elements.
30   The term "transgene" refers to a particular
nucleic acid sequence encoding a polypeptide or a
portion of a polypeptide to be expressed in a cell into
which the nucleic acid sequence is inserted. The term
"transgene" is meant to include (1) a nucleic acid
35 sequence that is not naturally found in the cell (*i.e.*,
a heterologous nucleic acid sequence); (2) a nucl ic

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acid sequence that is a mutant form of a nucleic acid
sequence naturally found in the cell into which it has
been inserted; (3) a nucleic acid sequence that serves
to add additional copies of the same (i.e., homologous)

5   or a similar nucleic acid sequence naturally occurring
in the cell into which it has been inserted; or (4) a
silent naturally occurring or homologous nucleic acid
sequence whose expression is induced in the cell into
which it has been inserted. By "mutant form" is meant a

10   nucleic acid sequence that contains one or more
nucleotides that are different from the wild-type or
naturally occurring sequence, i.e., the mutant nucleic
acid sequence contains one or more nucleotide
substitutions, deletions, and/or insertions. In some

15   cases, the transgene may also include a sequence
encoding a leader peptide or signal sequence such that
the transgene product will be secreted from the cell.

        The term "promoter" refers to a nucleic acid
sequence that regulates, either directly or indirectly,

20   the transcription of a corresponding nucleic acid coding
sequence to which it is operably linked. The promoter
may function alone to regulate transcription, or, in
some cases, may act in concert with one or more other
regulatory sequences such as an enhancer or silencer to

25   regulate transcription of the transgene.

        The term " an HCR enhancer" refers to a non-
coding nucleic acid sequence naturally located on human
chromosome 19 within or proximal to the apoE/apoC-I gene
locus, downstream of the ApoE and ApoC-I promoter/gene

30   sequences, but upstream of the ApoC-I pseudogene
sequence. As used herein, an HCR enhancer refers to any
nucleic acid sequence of about 774 base pairs, and to
fragment(s) thereof that has (have) biological activity.
When an HCR enhancer is operably linked to both a

35   promoter and a transgene, the HCR enhancer can (1)
confer a significant degree of liver specific expression

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of the transgene, and/or (2) can increase the level of
expression of the transgene in the liver.

     The term "rodent" refers to all members of the
phylogenetic order *Rodentia*, such as, for example,
5 mouse, rat, hamster, squirrel, or beaver.

     The term "progeny" refers to all offspring of
the transgenic mammal, and includes every generation
subsequent to the originally transformed transgenic
mammal.

10

Preparation of the Invention

     1. Preparation of DNA Constructs

15   A. Selection of Transgene

     This invention contemplates expression of one
or more transgenes primarily in the liver and/or the
gastro-intestinal tissue of a transgenic mammal. Where
20 the transgene is expressed primarily in the liver, the
gene product may be secreted into the bloodstream after
synthesis. Thus, included within the scope of this
invention is any transgene encoding a polypeptide to be
circulated in the blood. Typically, the transgene will
25 be a nucleic acid molecule encoding a polypeptide
involved in the immune response, hematopoiesis,
inflammation, cell growth and proliferation, cell
lineage differentiation, and/or the stress response.
The transgene may be homologous or heterologous to the
30 promoter and/or to the mammal. In addition, the
transgene may be a full length cDNA or genomic DNA
sequence, or any fragment, subunit or mutant thereof
that has at least some biological activity. Optionally,
the transgene may be a hybrid nucleic acid sequence,
35 *i.e.*, one constructed from homologous and/or
heterologous cDNA and/or genomic DNA fragments. The

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transgene may also optionally be a mutant of one or more
naturally occurring cDNA and/or genomic sequnces.

The transgene may be isolated and obtained in
suitable quantity using one or more methods that are
5 well known in the art. These methods and others useful
for isolating a transgene are set forth, for example, in
Sambrook et al. (Molecular Cloning: A Laboratory Manual,
Cold Spring Harbor Laboratory Press, Cold Spring Harbor,
NY [1989]) and in Berger and Kimmel (Methods in
10 Enzymology: Guide to Molecular Cloning Techniques, vol.
152, Academic Press, Inc., San Diego, CA [19AFM]).

Where the nucleic acid sequence of the
transgene is known, the transgene may be synthesized, in
whole or in part, using chemical synthesis methods such
15 as those described in Engels et al. (Angew. Chem. Int.
Ed. Engl., 28:716-734 [1989]). These methods include,
inter alia, the phosphotriester, phosphoramidite and H-
phosphonate methods of nucleic acid synthesis.

Alternatively, the transgene may be obtained
20 by screening an appropriate cDNA or genomic library
using one or more nucleic acid probes (oligonucleotides,
cDNA or genomic DNA fragments with an acceptable level
of homology to the transgene to be cloned, and the like)
that will hybridize selectively with the transgene DNA.
25   Another suitable method for obtaining a
transgene is the polymerase chain reaction (PCR).
However, successful use of this method requires that
enough information about the nucleic acid sequence of
the transgene is known so as to design suitable
30 oligonucleotide primers useful for amplification of the
appropriate nucleic acid sequence.

Where the method of choice requires the use of
oligonucleotide primers or probes (e.g. PCR, cDNA or
genomic library screening), the oligonucleotide
35 sequences selected as probes or primers should be of
adequate length and sufficiently unambiguous so as to

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minimize the amount of non-specific binding that will
occur during library screening or PCR. The actual
sequence of the probes or primers is usually based on
conserved or highly homologous sequences or regions from
5   the same or a similar gene from another organism.
Optionally, the probes or primers can be degenerate.

In cases where only the amino acid sequence of
the transgene is known, a probable and functional
nucleic acid sequence may be inferred for the transgene
10   using known and preferred codons for each amino acid
residue. This sequence can then be chemically
synthesized.

This invention contemplates the use of
transgene mutant sequences. A mutant transgene is a
15   transgene containing one or more nucleotide
substitutions, deletions, and/or insertions as compared
to the wild type sequence. The nucleotide substitution,
deletion, and/or insertion can give rise to a gene
product (*i.e.*, protein) that is different in its amino
20   acid sequence from the wild type amino acid sequence.
Preparation of such mutants is well known in the art,
and is described for example in Wells *et al.* (*Gene,*
34:315 [1985]), and in Sambrook *et al, supra.*

Preferred transgenes of the present invention
25   are erythropoietin (EPO), interleukin 1 (IL-1),
interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin
4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6),
interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin
9 (IL-9), interleukin 10 (IL-10), interleukin 11 (IL-
30   11), interleukin 12 (IL-12), ENA-78 (Walz *et al.,
J. Exp. Med.*, 174:1355-1362 [1991]; Strieter *et al.,
Immunol. Invest.*, 21:589-596 [1992]), interferon-α,
interferon-β, interferon-γ, granulocyte-colony stimulating
factor (G-CSF), granulocyte-macrophage colony
35   stimulating factor (GM-CSF), macrophage colony
stimulating factor (M-CSF), stem cell factor (SCF),

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keratinocyte growth factor (KGF), monocyte
chemoattractant protein-1 (MCP-1; Furutani et al.,
supra), tumor necrosis factor (TNF), AFM, and fragments,
subunits or mutants thereof. More preferred transgenes
5   include erythropoietin, interleukin 8, MCP-1,
keratinocyte growth factor, AFM, and ENA-78. The most
preferred transgenes include human interleukin 8, human
keratinocyte growth factor, AFM, and MCP-1.

10   B. Selection of Regulatory Elements

        This invention contemplates the use of
promoters that are regulated at least in part by an HCR
enhancer which results in increased liver expression of
15   the transgene.
        The promoter may be homologous (i.e., from the
same species as the mammal to be transfected with the
transgene) or heterologous (i.e., from a source other
than the species of the mammal to be transfected with
20   the transgene). As such, the source of the promoter may
be any unicellular prokaryotic or eukaryotic organism,
any vertebrate or invertebrate organism, or any plant,
provided that the promoter is functional in combination
with an HCR enhancer. The more preferred promoters of
25   this invention are the ApoA-I promoter, the ApoA-II
promoter, the ApoA-IV promoter, the ApoB promoter, the
ApoC-I promoter, the ApoC-II promoter, the ApoC-III
promoter, the ApoE promoter, the albumin promoter, the
alpha feto protein promoter, the PEPCK (phosphoenol
30   pyruvate carboxykinase) promoter (EP 365,591, published
May 2, 1990), the transthyretin promoter, the SV40
promoter, the CMV promoter, and the TK (thymidine
kinase) promoter. The most preferred promoters of this
group are ApoE, ApoC-I, and ApoA-IV. The most preferred
35   promoters are human ApoE and human ApoC-I.

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The preferred HCR enhancer element
contemplated herein is a non-coding DNA sequence located
on human chromosome 19 within, or proximal to, the
apoE/apoC-I gene locus, downstream of the ApoE and ApoC-

5   I genes, but upstream of the ApoC-I pseudogene. The
approximately 774 base pair HCR has been deposited under
the Budapest Treaty with the American Type Culture
Collection (ATCC; 12301 Parklawn Drive, Rockville, MD
20852) as accession number 69422. The date of deposit

10 is September 17, 1993. Fragments of this HCR sequence
are also contemplated herein, provided that the fragment
has the property of modulating expression of a transgene
in the liver (i.e., is biologically active).

The promoter sequences of this invention may
15 be obtained by any of several methods well known in the
art. Typically, promoters useful herein will have been
previously identified by mapping and/or by restriction
endonuclease digestion and can thus be isolated from the
proper tissue source using the appropriate restriction

20 endonucleases. In some cases, the promoter may have
been sequenced. For those promoters whose DNA sequence
is known, the promoter may be synthesized using the
methods described above for transgene synthesis.

Where all or only portions of the promoter
25 sequence are known, the promoter may be obtained using
PCR and/or by screening a genomic library with suitable
oligonucleotide and/or promoter sequence fragments from
the same or another species.

Where the promoter sequence is not known, a
30 fragment of DNA containing the promoter may be isolated
from a larger piece of DNA that may contain, for
example, a coding sequence or even another gene or
genes. Isolation may be accomplished by restriction
endonuclease digestion using one or more carefully

35 selected enzymes to isolate the proper DNA fragment.
After digestion, the desired fragment is isolated by

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agarose gel purification, Qiagen column or other methods
known to the skilled artisan. Selection of suitable
enzymes to accomplish this purpose will be readily
apparent to one of ordinary skill in the art.

5

C. Selection of Other Vector Components

In addition to the transgene, the promoter,
and the HCR enhancer, the vectors useful in this
10   invention typically contain one or more other elements
useful for (1) optimal functioning of the vector in the
mammal into which the vector is transfected, and (2)
amplification of the vector in bacterial or mammalian
host cells. Each of these elements will be positioned
15   appropriately in the vector with respect to each other
element so as to maximize their respective activities.
Such positioning is well known to the ordinary skilled
artisan. The following elements may be optionally
included in the vector as appropriate.

20

i. Signal Sequence Element

For those embodiments of the invention where
25   the transgene is to be secreted, a signal sequence, is
frequently present to direct the polypeptide encoded by
the transgene out of the cell where it is synthesized.
Typically, the signal sequence is positioned in the
coding region of the transgene towards or at the 5' end
30   of the coding region. Many signal sequences have been
identified, and any of them that are functional in the
transgenic tissue may be used in conjunction with the
transgene. Therefore, the signal sequence may be
homologous or heterologous to the transgene, and may be
35   homologous or heterologous to the transgenic mammal.
Additionally, the signal sequence may be chemically

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synthesized using methods set forth above. However, for
purposes herein, preferred signal sequences are those
that occur naturally with the transgene (i.e., are
homologous to the transgene).

5

ii. Membrane Anchoring Domain Element

In some cases, it may be desirable to have a
transgene expressed on the surface of a particular
10   intracellular membrane or on the plasma membrane.
Naturally occurring membrane proteins contain, as part
of the translated polypeptide, a stretch of amino acids
that serve to anchor the protein to the membrane.
However, for proteins that are not naturally found on
15   the membrane, such a stretch of amino acids may be added
to confer this feature. Frequently, the anchor domain
will be an internal portion of the protein and thus will
be engineered internally into the transgene. However,
in other cases, the anchor region may be attached to the
20   5' or 3' end of the transgene. Here, the anchor domain
may first be placed into the vector in the appropriate
position as a separate component from the transgene. As
for the signal sequence, the anchor domain may be from
any source and thus may be homologous or heterologous
25   with respect to both the transgene and the transgenic
mammal. Alternatively, the anchor domain may be
chemically synthesized using methods set forth above.

iii. Origin of Replication Element

30

This component is typically a part of
prokaryotic expression vectors purchased commercially,
and aids in the amplification of the vector in a host
cell. If the vector of choice does not contain an
35   origin of replication site, one may be chemically

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synthesized based on a known sequence, and ligated into
the vector.

5

               iv. <u>Transcription Termination Element</u>

          This element is typically located 3' to the
10   transgene coding sequence and serves to terminate
     transcription of the transgene. Usually, the
     transcription termination element is a polyadenylation
     signal sequence. While the element is easily cloned
     from a library or even purchased commercially as part of
15   a vector, it can also be readily synthesized using
     methods for nucleic acid synthesis such as those
     described above.

               v. <u>Intron Element</u>
20

          In many cases, transcription of the transgene
     is increased by the presence of one or more introns on
     the vector. The intron may be naturally occurring
     within the transgene sequence, especially where the
25   transgene is a full length or a fragment of a genomic
     DNA sequence. Where the intron is not naturally
     occurring within the DNA sequence (as for most cDNAs),
     the intron(s) may be obtained from another source. The
     intron may be homologous or heterologous to the
30   transgene and/or to the transgenic mammal. The position
     of the intron with respect to the promoter and the
     transgene is important, as the intron must be
     transcribed to be effective. As such, where the
     transgene is a cDNA sequence, the preferred position for
35   the intron is 3' to the transcription start site, and 5'
     to the polyA transcription termination sequence.

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Preferably for cDNA transgenes, the intron will be
located on one side or the other (i.e., 5' or 3') of the
transgene sequence such that it does not interrupt the
transgene sequence. Any intron from any source,

5   including any viral, prokaryotic and eukaryotic (plant
or animal) organisms, may be used to practice this
invention, provided that it is compatible with the host
cell(s) into which it is inserted. Also included herein
are synthetic introns. Optionally, more than one intron

10   may be used in the vector. A preferred intron is intron
1 of the human ApoE gene.

vi. <u>Selectable Marker(s) Element</u>

15   Selectable marker genes encode proteins
necessary for the survival and growth of transfected
cells grown in a selective culture medium. Typical
selection marker genes encode proteins that (a) confer
resistance to antibiotics or other toxins, e.g.,

20   ampicillin, tetracycline, or kanomycin for prokaryotic
host cells, and neomycin, hygromycin, or methotrexate
for mammalian cells; (b) complement auxotrophic
deficiencies of the cell; or (c) supply critical
nutrients not available from complex media, e.g., the

25   gene encoding D-alanine racemase for cultures of
Bacilli.

         All of the elements set forth above, as well
as others useful in this invention, are well known to

30   the skilled artisan and are described, for example, in
Sambrook et al.(Molecular Cloning: A Laboratory Manual,
Cold Spring Harbor Laboratory Press, Cold Spring Harbor,
NY [1989]) and Berger et al., eds. (Guide to Molecular
Cloning Techniques, Academic Press, Inc., San Diego, CA

35   [19AFM]).

- 20 -

D. Construction of Vectors

The vectors most useful in practicing this
invention are those that are compatible with prokaryotic
5   cell hosts. However, eukaryotic cell hosts, and vectors
compatible with these cells, are within the scope of the
invention.
In certain cases, some of the various vector
elements may be already present in commercially
10   available vectors such as pUC18, pUC19, pBR322, the pGEM
vectors (Promega Corp, Madison, WI), the pBluescript®
vectors such as pBIISK+/- (Stratagene Corp., La Jolla,
CA), and the like, all of which are suitable for
prokaryotic cell hosts.
15   However, where one or more of the elements are
not already present in the vector to be used, they may
be individually obtained and ligated into the vector.
Methods used for obtaining each of the elements are well
known to the skilled artisan and are comparable to the
20   methods set forth above for obtaining a transgene (i.e.,
synthesis of the DNA, library screening, and the like).
Preferred vectors of this invention are the
pGEM and the pBluescript® vectors. The most preferred
vector is pBIISK+.
25   Vectors used for amplification of the
transgene and/or for transfection of the mammalian
embryos are constructed using methods well known in the
art. Such methods include, for example, the standard
techniques of restriction endonuclease digestion,
30   ligation, agarose and acrylamide gel purification of DNA
and/or RNA, column chromatography purification of DNA
and/or RNA, phenol/chloroform extraction of DNA, DNA
sequencing, polymerase chain reaction amplification, and
the like, as set forth in Sambrook et al., supra.
35   The final vector used to practice this
invention is typically constructed from a starting

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v ctor such as a commercially available vector. This
vector may or may not contain some of the elements to be
included in the completed vector. If none of the
desired elements are present in the starting vector,
5   each element may be individually ligated into the vector
by cutting the vector with the appropriate restriction
endonuclease(s) such that the ends of the element to be
ligated in and the ends of the vector are compatible for
ligation. In some cases, it may be necessary to "blunt"
10   the ends to be ligated together in order to obtain a
satisfactory ligation. Blunting is accomplished by
first filling in "sticky ends" using Klenow DNA
polymerase or T4 DNA polymerase in the presence of all
four nucleotides. This procedure is well known in the
15   art and is described for example in Sambrook et al.,
supra.

Alternatively, two or more of the elements to
be inserted into the vector may first be ligated
together (if they are to be positioned adjacent to each
20   other) and then ligated into the vector.

One other method for constructing the vector
to conduct all ligations of the various elements
simultaneously in one reaction mixture. Here, many
nonsense or nonfunctional vectors will be generated due
25   to improper ligation or insertion of the elements,
however the functional vector may be identified and
selected by restriction endonuclease digestion.

After the vector has been constructed, it may
be transfected into a prokaryotic host cell for
30   amplification. Cells typically used for amplification
are E coli DH5-alpha (Gibco/BRL, Grand Island, NY) and
other E. coli strains with characteristics similar to
DH5-alpha.

Where mammalian host cells are used, cell
35   lines such as Chinese hamster ovary (CHO cells; Urlab et
al., Proc. Natl. Acad. Sci USA, 77:4216 [1980])) and

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human embryonic kidney cell line 293 (Graham et al., J. Gen. Virol., 36:59 [1977]), as well as other lines, are suitable.

5   Transfection of the vector into the selected host cell line accomplished using such methods as calcium phosphate, electroporation, microinjection, lipofection or DEAE-dextran method. The method selected will in part be a function of the type of host cell to be transfected. These methods and other suitable

10   methods are well known to the skilled artisan, and are set forth in Sambrook et al., supra.

      After culturing the cells long enough for the vector to be sufficiently amplified (usually overnight for E. coli cells), the vector (often termed plasmid at

15   this stage) is isolated from the cells and purified. Typically, the cells are lysed and the plasmid is extracted from other cell contents. Methods suitable for plasmid purification include inter alia, the alkaline lysis mini-prep method (Sambrook et al.,

20   supra).

      E. Preparation of Plasmid For Insertion into the Embryo

      Typically, the plasmid containing the

25   transgene is linearized using a selected restriction endonuclease prior to insertion into the embryo. In some cases, it may be preferable to isolate the transgene, promoter, and regulatory elements as a linear fragment from the other portions of the vector, thereby

30   injecting only a linear nucleic acid sequence containing the transgene, promoter, intron (if one is to be used), enhancer, polyA sequence, and optionally a signal sequence or membrane anchoring domain into the embryo. This may be accomplished by cutting the plasmid so as to

35   remove the nucleic acid sequence region containing these

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elements, and purifying this region using agarose gel
electrophoresis or other suitable purification methods.

5

2. Production of Transgenic Mammals

Transgenic mammals may be prepared using
10   methods well known to the skilled artisan. For example,
to prepare transgenic rodents such as mice, methods such
as those set forth by Hogan *et al.*, eds. (*Manipulating
The Mouse Embryo: A Laboratory Manual*, Cold Spring
Harbor Laboratory Press, Cold Spring Harbor, New York
15   [1986]) may be employed.
The specific line(s) of any mammalian
species used to practice this invention are selected for
general good health, good embryo yields, good pronuclear
visibility in the embryos, and good reproductive
20   fitness. For example, when transgenic mice are to be
produced, lines such as C57/BL6 x DBA2 F1 cross, or FVB
lines are often used (obtained commercially from Charles
River Labs, Boston, MA). The line(s) used to practice
this invention may themselves be transgenics, and/or may
25   be knockouts (*i.e.*, mammals which have one or more genes
partially or completely suppressed).
The age of the mammals that are used to obtain
embryos and to serve as surrogate hosts is a function of
the species used, but is readily determined by one of
30   ordinary skill in the art. For example, when mice are
used, pre-puberal females are preferred, as they yield
more embryos and respond better to hormone injections.
Similarly, the male mammal to be used as a
stud will normally be selected by age of sexual
35   maturity, among other criteria.

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Administration of hormones or other chemical
compounds may be necessary to prepare the female for egg
production, mating, and/or reimplantation of embryos.
The type of hormones/cofactors and the quantity used, as
5   well as the timing of administration of the hormones
will vary for each species of mammal. Such
considerations will be readily apparent to one of
ordinary skill in the art

Typically, a primed female (i.e., one that is
10 producing eggs that can be fertilized) is mated with a
stud male, and the resulting fertilized embryos are then
removed for introduction of the transgene(s).
Alternatively, eggs and sperm may be obtained from
suitable females and males and used for in vitro
15 fertilization to produce an embryo suitable for
introduction of the transgene.

Normally, fertilized embryos are incubated in
suitable media until the pronuclei appear. At about
this time, exogenous nucleic acid comprising the
20 transgene of interest is introduced into the female or
male pronucleus. In some species such as mice, the male
pronucleus is preferred.

Introduction of nucleic acid may be
accomplished by any means known in the art such as, for
25 example, microinjection, electroporation, or
lipofection. Following introduction of the transgene
nucleic acid sequence into the embryo, the embryo may be
incubated in vitro for varying amounts of time, or
reimplanted into the surrogate host, or both. In vitro
30 incubation to maturity is within the scope of this
invention. One common method is to incubate the embryos
in vitro for about 1-7 days, depending on the species,
and then reimplant them into the surrogate host.

Reimplantation is accomplished using standard
35 methods. Usually, the surrogate host is anesthetized,
and the embryos are inserted into the oviduct. The

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number of embryos implanted into a particular host will
vary by species, but will usually be comparable to the
number of offspring the species naturally produces.

5   Transgenic offspring of the surrogate host may
be screened for the presence and/or expression of the
transgene by any suitable method. Screening is often
accomplished by Southern blot or Northern blot analysis,
using a probe that is complementary to at least a
portion of the transgene. Western blot analysis using
10   an antibody against the protein encoded by the transgene
may be employed as an alternative or additional method
for screening for the presence of the transgene product.
Typically, DNA is prepared from tail tissue (about 1 cm
is removed from the tip of the tail) and analyzed by
15   Southern analysis or PCR for the transgene.
Alternatively, the tissues or cells believed to express
the transgene at the highest levels are tested for the
presence and expression of the transgene using Southern
analysis or PCR, although any tissues or cell types may
20   be used for this analysis.

Alternative or additional methods for
evaluating the presence of the transgene include,
without limitation, suitable biochemical assays such as
enzyme and/or immunological assays, histological stains
25   for particular markers or enzyme activities, and the
like. Analysis of the blood may also be useful to
detect the presence of the transgene product in the
blood, as well as to evaluate the effect of the
transgene on the levels of various types of blood cells
30   and other blood constituents.

Progeny of the transgenic mammals may be
obtained by mating the transgenic mammal with a suitable
partner, or by *in vitro* fertilization of eggs and/or
sperm obtained from the transgenic mammal. Where mating
35   with a partner is to be performed, the partner may or
may not be transgenic and/or a knockout; where it is

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transgenic, it may contain the same or a different
transgene, or both. Alternatively, the partner may be a
parental line. Where *in vitro* fertilization is used,
the fertilized embryo may be implanted into a surrogate
5   host or incubated *in vitro*, or both. Using either
method, the progeny may be evaluated for the presence of
the transgene using methods described above, or other
appropriate methods.

        The transgenic mammals of this invention may
10   be used to generate one or more cell lines. Such cell
lines have many uses, as for example, to evaluate the
effect(s) of the transgene on a particular tissue or
organ, and to screen compounds that may affect the level
of activity of the transgene in the tissue. Such
15   compounds may be useful as therapeutics to modulate the
activity of the transgene.

        Production of cell lines may be accomplished
using a variety of methods, known to the skilled
artisan. The actual culturing conditions will depend on
20   the tissue and type of cells to be cultured. Various
media containing different concentrations of macro and
micro nutrients, growth factors, serum, and the like,
can be tested on the cells without undue experimentation
to determine the optimal conditions for growth and
25   proliferation of the cells. Similarly, other culturing
conditions such as cell density, media temperature, and
carbon dioxide concentrations in the incubator can also
readily be evaluated.

        The transformed mammals, their progeny, and
30   transgenic cell lines of the present invention provide
several important uses that will be readily apparent to
one of ordinary skill in the art. The mammals and cell
lines are particularly useful for (a) providing and
evaluating the potential of treatments (such as gene
35   therapy) for a variety of conditions and diseases,
and/or (b) screening compounds that have potential as

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prophylactics or therapeutics. Such uses may be found
for (1) conditions caused by inflammation, (2) immune
system disorders, (3) epithelial cell repair (skin, lung
and/or intestinal epithelia), (4) hematopoiesis, and/or

5   (5) disorders caused by various physical and/or mental
stresses. For example, transgenic mammals or cell lines
containing the transgene for IL-8 will be useful for
identifying compounds that modulate neutrophil
migration; transgenic mammals containing the transgene

10 KGF will be useful for evaluating epithelial tissue
repair, and identifying compounds that affect this
process.

In the case of transgenic mammals, screening
of candidate compounds is conducted by administering the

15 compound(s) to be tested to the mammal, over a range of
doses, and evaluating the mammal's physiological
response to the compound(s) over time. Administration
may be by any appropriate means such as, for example,
oral administration, or administration by injection,

20 implantation, or transdermal delivery, depending on the
chemical nature of the compound being evaluated. In
some cases, it may be appropriate to administer the
compound in conjunction with other compounds or co-
factors that might enhance the efficacy of the compound.

25 In screening cell lines for compounds useful
in treating the above mentioned problems, the compound
is added to the cell culture medium at the appropriate
time, and the cellular response to the compound is
evaluated over time using the appropriate biochemical

30 and/or histological assays. In some cases, it may be
appropriate to apply the compound of interest to the
culture medium in conjunction with other compounds or
co-factors that might enhance the efficacy of the
compound.

35

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The invention will be more fully understood by
reference to the following examples. They should not be
construed in any way as limiting the scope of the
present invention.

5

EXAMPLES

10   Example 1: Preparation of a HCR-IL-8 Transgenic Mouse

A. Construction of Transgene and Vectors

A diagram depicting the overall cloning strategy
15   used herein is set forth in Figure 2.
A PstI-XbaI DNA fragment of about 774 base pairs
(containing HCR sequence) obtained from the human apoC-
I/C-I' intergenic region on chromosome 19 (Simonet
et al.[1993], supra) was subcloned into the PstI-XbaI
20   sites of pUC19 (New England Biolabs, Beverly, MA). The
resulting plasmid was designated pCI-CI'PX#8. This
plasmid has been deposited on September 17, 1993 with
the ATCC as accession number 69422.
An approximately 1.45 kb Kpn-I fragment containing
25   a contiguous piece of DNA consisting of 650 bp of the
human ApoE gene 5'-flanking sequence, the first exon,
first intron and a portion of the second exon of the
ApoE gene was excised from the vector pHE54 (Simonet et
al., [1993], supra). This fragment of about 1.45 kb was
30   inserted by ligation into the Kpn-I cloning site of pCI-
CI'PX#8. After ligation, the plasmid was transfected
into E coli strain DH5-alpha (Gibco/BRL, Grand Island,
NY). The cells were plated out on standard LB (Luria
broth) or TB (Terrific broth) plus ampicillin medium
35   (Sambrook et al., supra) on agarose plates, and grown up
overnight at 37°C.

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Colonies were then selected and grown up overnight
in standard LB medium in the presence of ampicillin for
amplification. After amplification, plasmid DNA from
each amplified colony was prepared using the standard
5   alkaline lysis miniprep method (Sambrook *et al., supra*),
and the plasmid DNA was purified using a Qiagen column
(Qiagen Corp., Chatsworth, CA). Purified plasmid was
then digested with the restriction endonuclease BamHI
and analyzed by agarose gel electrophoresis. Of
10   18 colonies analyzed, 6 were found to have a single
insert ligated in the desired orientation. The
resulting construct containing the HCR upstream of the
ApoE promoter and exon/intron sequence was designated
pHCR-HEP.
15   The approximately 2.2 kb HCR enhancer-promoter-
intron cassette was excised from pHCR-HEP as either a
PstI-EcoRI fragment or a *HindIII-EcoRI* fragment. Each
of these fragments were ligated into pBIISK+ (Stratagene
Corp., La Jolla, CA) to generate the plasmids pHCR-HEP
20   BS (PE) (*PstI-EcoRI* fragment) and pHCR-HEP BS (HE)
(*HindIII-EcoRI* fragment).
The eukaryotic expression vector V19-10 was used as
a template for amplification of the SV40 polyA+ signal.
This vector was constructed by inserting a 592 base pair
25   *AatII/ClaI* fragment containing the origin of replication
sequence from bacteriophage M13 into the eukaryotic
expression vector V19-8 (described in WO 91/05795,
published May 2, 1991). The 242 base pair polyA+
sequence from V19-10 was amplified as a *NotI-SacII*
30   fragment or a *HindIII-XhoI* fragment using PCR. The
primers used for PCR amplification were:

NotI-SacII fragment:

35   Primer 1:   CTCTAGAAAGCTTAATTCAGTC (SEQ ID NO: 2)

- 30 -

Primer 2:   TCCCCGCGGGGAAGAGCGCAGAGCTCGG (SEQ ID NO: 3)

Thirty cycles of amplification were conducted as
follows: Denaturation was at 94°C for 30 seconds;
5   annealing was at 56°C for 30 seconds; and extension was
at 72°C for 30 seconds.

HindIII-XhoI fragment:

10   Primer 3:   CTCTAGAAAGCTTAATTCAGTC (SEQ ID NO: 4)

Primer 4:   CTGGATCTCGAGGTACCCGGGGATCATAATC (SEQ ID NO: 5)

Thirty cycles of amplification were conducted as
15   follows: Denaturation was at 94°C for 30 seconds;
annealing was at 57°C for 30 seconds; and extension was
at 72°C for 30 seconds.
The PCR fragments were sequenced and showed 100%
homology to the template. The fragments were then
20   subcloned into _NotI-SacII_ cut or _HindIII-XhoI_ cut
pBIISK+, to generate the plasmids pBS-PA (NS) and pBS-PA
(HX), respectively.
The human IL-8 cDNA was obtained by screening a
human peripheral blood lymphocyte cDNA library, prepared
25   as follows:
Peripheral blood lymphocytes were isolated from
freshly prepared buffy coats, on a ficol-paque step
gradient (Pharmacia, Uppsala, Sweden). Mononuclear
cells present in the interphase of the gradient were
30   removed and washed with PBS three times. The cells were
then suspended in the medium RPMI 1640 + 10% FCS (fetal
calf serum). About 5 million cells/ml were incubated
with pokeweed mitogen (10 ug/ml, Sigma Chemical Corp.,
St. Louis, MO) for 19 hours, followed by addition of
35   cycloheximide to a final concentration of 10 ug/ml for

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an additional 6 hours.   Incubation was carried out at
37°C and 5% CO_2.

        Total RNA was isolated from activated lymphocytes
using the guanidium thiocyanate-CsCl technique (Chirgwin
5   et al., *Biochem.*, 18: 5294-5299 [1979]).   Polyadenylated
RNA was selected by oligo(dT) chromatography.   The
polyA+ RNA was then ethanol precipitated and
centrifuged.   The final pellet was dissolved in water
and kept in liquid nitrogen in aliquots.

10   About 5 ug of polyA+ RNA were used for cDNA library
construction.   After denaturation with methyl mercury
hydroxide, oligo(dT)-primed double strand cDNA was
synthesized following the procedure set forth in
Sambrook *et al.*, *supra*, followed by methylation with
15 *EcoRI* and Alu methylases.   The technique of Dorssers *et
al*, (*Nuc. Acid. Res.*, 15: 3629, [19AFM]) was used to
introduce *EcoRI* and *HindIII* sites on the 5' and 3' ends
of the cDNAs, respectively.   After digestion with *EcoRI*
and *HindIII* restriction enzymes, cDNAs that were larger
20 than 500 base pairs were isolated from an agarose gel by
electroelution.   The eukaryotic expression vector V19-10
(described above), was digested with *EcoRI* and *HindIII*
and was then ligated with the cDNAs.   These new plasmids
containing cDNA inserts were transfected into competent
25 DH5 alpha cells (GIBCO-BRL, Gaithersburg, MD).   The cDNA
library was frozen in aliquots at -80°C after addition
of DMSO to 7% (Okayama & Berg, *Mol. Cell. Biol.*, 2: 161-
170, 1982).

        A mixed oligonucleotide probe was designed on the
30 basis of similarity in nucleotide sequences surrounding
and coding for the signal peptidase cleavage site of a
number of cytokines.   The sequence of this degenerate
probe was:

35   ATGTCGACMWCSVTGCMCCHRYMYSMYCYA   (SEQ ID NO: 6)

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In this sequence, M, W, S, V, R, Y, and H represent degenerate nucleotides. M represents A or C; W represents A or T; S represents C or G; V represents A or C or G; R represents A or G; Y represents C or T; and

5   H represents A or C or T.

Using this probe, a cDNA encoding IL-8 was obtained and sequenced for homology comparison to the published sequence for IL-8 (Furutani et al., Biophys. Biochem.

10   Res. Comm., 159:249-255 [1989]). The IL-8 cDNA clone was then used as a template to PCR amplify a SpeI-NotI fragment of the cDNA. Amplification was accomplished using the following oligonucleotide primers:

15   **Primer 5**: GGACTAGTCCAGAGCACACAAGCTTCTAG   (SEQ ID NO: 7)

Primer 6: ATAAGAATGCGGCCGCTAAACTATTGCATCTGGCAACCC   (SEQ ID NO: 8)

Thirty cycles of amplification were conducted as

20   follows: Denaturation was at 94°C for 30 seconds; annealing was at 54°C for 30 seconds; and extension was at 72°C for 30 seconds.
The amplified fragment was then subcloned into *SpeI-NotI* cut pIIBS-PA (NS) to produce the plasmid pIL-8

25   PA. The amplified IL-8 sequence, which lacked a portion of the 3' untranslated sequence of the original IL-8 cDNA, was sequence verified and found to be 100% homologous to human IL-8 in the coding region.
The polyadenylated IL-8 cDNA was put under the

30   control of the HCR enhancer and the ApoE promoter by excising the HCR-ApoE promoter-intron cassette from the vector pHCR-HEP BS(HE) as a *XhoI-SpeI* fragment. This fragment was then subcloned into *XhoI-SpeI* cut pIL-8 PA to generate the plasmid pHCR-HEP IL-8 PA (abbreviated

35   HE8).

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For microinjection, the plasmid HE8 was digested
with restriction enzymes *XhoI*, *ScaI* and *AflIII*, and the
approximately 3.3 Kb *XhoI-AflIII* insert fragment
containing the HCR, the ApoE promoter, the ApoE first
5   exon, first intron, a portion of the second exon, the
human IL-8 cDNA and the SV40 poly-adenylation signal
was purified on a 0.8% ultrapure DNA agarose gel (BRL
Corp., Bethesda, MD) and diluted to 1 ng/ul in 5mM Tris,
pH 7.4, 0.2mM EDTA. About 2 to 3 picoliters of this
10   solution were injected into the male pronucleus of each
mouse embryo.

To prepare a liver expression vector to make
transgenic mammals containing the transgenes KGF or MCP-
1, the approximately 242 base pair *HindIII-XhoI* insert
15   fragment from pBS PA (HX) was isolated and subcloned
into *HindIII-XhoI* cut pHCR-HEP BS (PE). The resulting
vector, pliv(BSK), has a polylinker region containing
EcoRI, *EcoRV*, and *HindIII* restriction sites downstream
of the HCR-ApoE promoter-intron cassette and upstream of
20   the SV40 poly-adenylation signal.

B. Preparation of Embryos and Microinjection

Pregnant mare's serum ("PMS"), supplying
25   Follicle Stimulating Hormone ("FSH") was administered
to female mice of the strain BDF1 (Charles River Labs,
Boston, MA) about three days prior to the day of
microinjection. PMS (obtained from Sigma Chemicals) was
prepared as a 50 I.U./ml solution in Phosphate Buffered
30   Saline and injected intraperitoneally at 0.1 ml (5 I.U.)
per animal. Human Chorionic Gonadotropin ("HCG"),
supplying Luteinizing Hormone ("LH") was administered
45-48 hours after the PMS injections. HCG was also
prepared as a 50 I.U./ml solution in PBS and injected IP
35   (intraperitoneally) at 0.1 ml per animal. Females were
placed with stud males of the same strain immediately

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after HCG injections. After mating, the females were
examined for a vaginal copulation plug. The appearance
of an opaque white plug indicated a successful mating.

Successfully mated females were sacrificed by
5   cervical dislocation, and both oviducts were rapidly
removed and placed in M2 medium (Hogan et al., eds.,
Manipulating the Mouse Embryo: A Laboratory Manual,
Cold Spring Harbor Laboratory Press, pp 249-257 [1986]).
The oviducts were transferred individually from M2
10   medium to PBS containing 300 µg/ml hyaluronidase (Sigma
Corp., St. Louis, MO.) in a round bottom dissection
slide. The embryos were teased out of the oviduct and
allowed to settle at the bottom of the slide as the
cumulus cells detached from the embryos. When the
15   cumulus masses were disaggregated (about 5 minutes) the
embryos were transferred through two washes of M2 medium
and the fertilized embryos were separated from
unfertilized and abnormal embryos. The fertilized
embryos were then transferred through 5% CO_2
20   equilibrated M16 medium (Hogan et al., supra), placed in
equilibrated microdrop dishes containing M16 medium
under paraffin oil and returned to the incubator.

Fertilized single-cell embryos from BDF1
xBDF1-bred mice were selected in M16 medium and
25   incubated about 5 hours at 37°C until the pronuclei
appeared. Embryos were then transferred into M2 medium
in a shallow depression slide under paraffin oil and
placed under the microscope. The pronuclei were easily
visible under 200X magnification. Using suction on the
30   holding pipet, a single embryo was selected and rotated
such that the male pronucleus was away from the holding
pipet. Approximately 2 to 3 picoliters of solution
containing the DNA construct at about 1 microgram per ml
was injected into one of the pronuclei, preferably the
35   male pronucleus. Following the injection, the embryos

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were returned to incubation for 18 hours and reimplanted
the next day into foster pseudopregnant femal s.

Reimplantations were performed on anesthetized
female mice of strain CD1 using a dissecting microscope.
5   A pseudo-pregnant female mouse was anaesthetized with
0.017-0.020 ml/g body weight of avertin, injected IP.
The mouse was placed under the dissecting microscope and
the incision area was disinfected with 70% ethanol. The
ovary was exteriorized and the bursal sac that surrounds
10   the ovary and the oviduct was carefully pulled open.
The ovary and oviduct were separated to expose the
opening of the oviduct (termed the infindibulum).
Surviving embryos were then removed from the incubator
and loaded into the reimplantation pipet. The tip of
15   the pipet was inserted several millimeters into the
infindibulum and gentle pressure was used to deliver the
embryos into the oviduct. About 10 to 20 2-cell embryos
were implanted per mouse, resulting in a litter size of
about 3 to 12. The ovary then was returned to the
20   peritoneum, and the body wall and then the skin were
sutured.

C. Identification of Transgenic Mice

25   Of 52 mice born after embryo injections, 9
contained the IL-8 transgene as assayed by PCR
amplification. About 1 cm of the tail of each mouse was
removed, and DNA was prepared using the technique set
forth by Hogan et al., supra. The DNA was then
30   subjected to PCR analysis using the following primers:

Primer 7:   GCCTCTAGAAAGAGCTGGGAC   (SEQ ID NO: 9)

Primer 8:   CGCCGTGTTCCATTTATGAGC   (SEQ ID NO: 10)

35

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The PCR amplification procedure was denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds. Thirty cycles were performed.

5   The resultant transgenic mice harboring the transgene in their genome are termed the founder mice. The founder mice were backcrossed to strain BDF1 mice to generate heterozygous F1 transgenic mice.

To evaluate the F1 transgenic mice for the presence
10 and effect of IL-8, blood was obtained and analyzed as follows.

Quantitation of serum IL-8 levels were determined using an Elisa kit for human IL-8 (obtained from Biosource International, Camarillo, CA) and following
15 the manufacturer's protocol. The results are shown in Figure 3A. As can be seen, three of the lines of F1 transgenic mice (HE8 lines 7, 26, and 51) had levels of about 100 ng/ml or higher, while no IL-8 was detected in the serum of the non-transgenic (NT) mice.

20 Circulating white blood cells in the serum of the F1 transgenic and non-transgenic mice were counted using a Sysmex F-800 blood cell counter (Toa Medical Electronics Co., LTD, Kobe, Japan) and following the manufacturer's protocol. Prior to counting, red blood
25 cells were lysed with Quicklyser™ (Toa Medical Electronics Co., LTD, Kobe, Japan), following the manufacturer's protocol. For differential leukocyte analysis, about 3 µl of whole blood were spread on a glass slide and subjected to Wright's-Giemsa staining.
30 At least 100 cells were counted from each slide by visualizing the cells under a 100x oil emersion lens on an Olympus CH2 student microscope. Neutrophils were distinguished from lymphocytes, macrophages, eosinophils, and basophils by their multinucleated
35 structures. For all lines reported, at least five individual F1 heterozygotes were bled and analyzed.

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Absolute neutrophil levels were determined by
multiplying the percentage of neutrophils on the
Wright's-Giemsa stained slides by the total white blood
cell count obtained from the Sysmex counter. The
5   results are shown in Figure 3B. Three of the F1
transgenic lines evaluated (HE8 lines 7, 26, and 51) had
a circulating neutrophil level of greater than 6,000/ µl
blood, while the non-transgenic (NT) mice had a level of
under 1,000/ µl blood.

10

Example 2: Preparation of a HCR-KGF Transgenic Mouse

The gene encoding human KGF (keratinocyte growth
factor) was obtained by PCR amplification of the gene
15   from a normal human dermal fibroblast cDNA library. PCR
amplification of KGF was accomplished using the
following two oligonucleotide primers:

Primer 9:   CAATCTACAATTCACAGA (SEQ ID NO: 11)

20

Primer 10:   TTAAGTTATTGCCATAGG (SEQ ID NO: 12)

The conditions for PCR were: denaturation at 92°C for 20
seconds; anneal at 55-40°C for 20 seconds (this
25   consisted of 2 cycles at 55°C, followed by 2 cycles at
45°C, which was followed by 28 cycles at 40°C); and
extension at 72°C for 30 seconds. Thirty cycles total
were performed.
To introduce HindIII and BglII restriction sites to
30   the ends of the KGF cDNA, the cDNA was PCR amplified
using the following two oligonucleotide primers:

Primer 11:   AACAAAGCTTCTACAATTCACAGATAGGA (SEQ ID NO: 13)

35   Primer 12:   AACAAGATCTTAAGTTATTGCCATAGG (SEQ ID NO: 14)

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The conditions for PCR were: denaturation at 92°C for 20
seconds; anneal at 45°C for 20 seconds; and elongation
at 72°C for 30 seconds. Thirty cycles were performed.

5   After amplification, the KGF cDNA was purified and
digested with *HindIII* and *BglII*, and then ligated into
the vector pCFM3006. This vector was prepared from the
vector pCFM836 (described in U.S. Patent No. 4,710,473,
issued December 1, 19AFM). The two endogenous *NdeI*
10   restriction sites in pCFM836 were removed by cutting
pCFM836 with *NdeI*, filling in the cut ends of the vector
using T4 polymerase, and then re-ligating the vector by
blunt end ligation. Next, the DNA sequence between the
AatII and *KpnI* sites of the now modified pCFM836 was
15   altered using the technique of PCR overlapping
oligonucleotide mutagenesis. The following changes at
the base pair positions listed were made (the base pair
position changes are relative to the *BglII* site on
pFM836 which is position #180):

20

plasmid bp #	bp changed
# 428	G/C
# 509	A/T
# 617	insert two G/C bp
# 978	C/G
# 992	A/T
# 1002	C/G
# 1005	T/A
# 1026	T/A
# 1045	T/A
# 1176	T/A
# 1464	T/A
# 2026	bp deletion
# 2186	T/A
# 2479	T/A
# 2498-2501	GTCA

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# 2641-2647	bp deletion
# 3441	A/T
# 3649	T/A

5   The KGF cDNA in this vector was used as a template
for amplification. A 710 base pair *HindIII* fragment of
KGF was amplified using PCR and the following two
oligonucleotide primers:

10   Primer 13: CGATCGTAAGCTTGGTCAATGACCTAGGAGTAAC (SEQ ID NO: 15)

     Primer 14: CGATCGTAAGCTTGCGGATCCTAAGTTATTGCC (SEQ ID NO: 16)

     Amplification was conducted for 30 cycles. Denaturation
15   was at 94°C for 30 seconds, annealing was at 58°C for 20
seconds, and elongation was at 72°C for 30 seconds. The
amplified fragment was purified by agarose gel
electrophoresis and then ligated into the vector plivBsk
(described in Example 1; shown in Figure 2). *E. coli*
20   cells were then transformed with the ligation mixture
and plated out for overnight incubation. After
incubation, colonies were selected, grown up, and the
plasmids analyzed for those containing KGF in the proper
orientation. The orientation of the plasmid KGF was
25   determined by restriction endonuclease digestion with
EcoRI. Clones with the proper orientation were grown up
and the plasmid purified using a Qiagen column (Qiagen
Corp., Chatsworth, CA). Several clones were sequenced
to verify the orientation and sequence of the KGF.
30   DNA to be used in microinjection of the embryos was
prepared by cutting the vector containing KGF with *SpeI*
and *XhoI* to obtain a DNA fragment containing (in order)
the HCR, ApoE promoter, KGF, and polyA sequences. This
DNA was gel purified and prepared as described in
35   Example 1. Microinjection and implantation into
pseudopregnant mice were as described in Example 1.

- 40 -

Example 3: Preparation of a HCR-MCP-1 Transgenic Mouse

        The cDNA encoding human MCP-1 was obtained by
5   screening the human peripheral blood lymphocyte library
    described in Example 1 with the following probe:

CTGTSYCTSCTSNTSMTWGTWGCYGSCT (SEQ ID NO: 17)

10 In the probe sequence, S represents C or G; Y represents
    T or C; N represents A or T or C or G; M represents C or
    A; and W represents A or T.
        A clone of about 850 base pairs was obtained
    using this probe and was inserted into the vector V19-8
15 (described in Example 1). This clone was then sequenced
    for identification, and found to be homologous to the
    published sequence for MCP-1 (Matsushima et al., *J. Exp.
    Med.*, 167:1883-1893 [1988]).
        The MCP-1 cDNA was excised from the vector
20 V19-8 as an approximately 350 base pair *EcoRI-PstI*
    fragment, and was ligated into the vector pUC19
    previously cut with *EcoRI* and *HindIII*. The cDNA was
    then removed as an *EcoRI-HindIII* fragment and inserted
    into the vector plivBSK. The vector containing the cDNA
25 was called HEMF. This vector was transformed into
    E. coli strain DH5 alpha for amplification. After
    culturing the cells overnight, the plasmid was isolated
    and purified using the alkaline lysis method, followed
    by cesium chloride centrifugation.
30   After centrifugation, the plasmid was digested
    with the restriction enzymes *SpeI*, *XhoI*, and *ScaI*, and
    the approximately 2.8 kilobase DNA fragment containing
    the HCR, ApoE promoter and first intron, MCP-1 cDNA, and
    the SV40 polyA sequence was isolated. This DNA was gel
35 purified and prepared for microinjection as described in
    Example 1. Microinjection of embryos and implantation

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of embryos into pseudopregnant mice were as described in
Example 1.

Of 130 offspring analyzed, 5 contained the
MCP-1 transgene as detected using PCR analysis.

Example 4: Preparation of a HCR-AFM Transgenic Mouse

The cDNA encoding human AFM was obtained as
follows:

The polymerase chain reaction (PCR) was used
to amplify a portion of the cDNA encoding AFM. PCR was
first performed in a total volume of about 100µl using
approximately one nanogram of Quick Clone human liver
cDNA (Clontech, cat. no. 7113-1) as the template and
standard PCR buffer (Perkin-Elmer Cetus). About 1 uM of
each of the following two degenerate primers was also
used in this PCR reaction.

ACGCTGAATTCGCCARAARTTYATHGARGAYAA (SEQ ID
NO:18)

ACGCTAAGCTTGCRTCYTTRTADATYTGNACDAT (SEQ ID
NO:19)

In these primer sequences, R represents A or
G; Y represents T or C; N represents A or T or C or G; D
represents G or A or T; H represents A or C or T.

The conditions used for the PCR reaction were
as follows: 95°C for 8 min (1 cycle); 94°C for 1 min,
34°C for 10 min and 72°C for 2 min (3 cycles); 94°C for
1 min, 50°C for 1 min, and 72°C for 2 min (45 cycles);
72°C for 5 min (1 cycle).

An approximately 1 µl aliquot of amplified DNA
obtained from this PCR was used as a template for a

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second PCR using a nested primer pair. For this second
PCR, the following degenerate primers were used:

ACGCTGAATTCGCGAYAAYATHGARTAYATHAC (SEQ ID
5   NO:20)

ACGCTAAGCTTGCNGARTAYTCRAANGTRAA (SEQ ID NO:21)

10   In these primer sequences, R represents A or
G; Y represents T or C; H represents A or C or T; N
represents A or T or C or G.

          This second PCR was performed using the same
15   reaction mix and cycling parameters as for the first
PCR. Analysis of this second PCR by agarose gel
electrophoresis revealed the amplification of an
approximately 1 kb DNA fragment. This DNA fragment was
gel purified, and then digested with restriction
20   endonucleases EcoRI and HindIII, and ligated into the
cloning/sequencing vector mp19 (Boehringer Mannheim
Corporation) for sequencing.
          An oligonucleotide identical to a small
portion of the sequence of the DNA fragment was
25   generated and used to isolate the full-length AFM cDNA
from a human liver cDNA library (Clonetech, cat no.
HL1115a). The sequence of this oligonucleotide was:

TATGTGCTATGGAGGGGC (SEQ ID NO:22)
30
          Positive clones from this library screening
were purified using standard procedures and then re-
screened with the same oligonucleotide probe, and a
single clone (called 17AFM) containing an approximately
35   2.3 kb insert was selected. This clone was inserted
into the vector pGem3Z for sequencing to confirm that it

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encoded the full-length cDNA for human AFM. The nucleic
acid sequence and translated amino acid sequence for
this clone are set forth in Figure 4.

5   To prepare a transgenic mouse containing the
transgene human AFM, the AFM cDNA clone was removed from
the vector by digesting with *EcoRI*. The cDNA was then
inserted into the vector plivBSK (described in Example
I). This vector containing the AFM cDNA, was
transformed into *E. coli* strain DH5 alpha for
10   amplification. After culturing the cells overnight, the
plasmid was isolated and purified using the standard
alkaline lysis method, followed by cesium chloride
centrifugation.

          After centrifugation, the plasmid was digested
15   with the restriction enzymes *SpeI*, *XhoI*, and *ScaI*, and
the DNA fragment containing the HCR, ApoE promoter and
first intron, AFM cDNA and the SV40 polyA sequence was
isolated. This DNA was gel purified and prepared for
microinjection as described in Example I.
20   Microinjection and implantation of embryos
into pseudopregnant mice were as described in Example I.

          All literature cited herein is expressly
incorporated by reference.
25

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Amgen Inc.

   (ii) TITLE OF INVENTION: Tissue Specific Transgene Expression

  (iii) NUMBER OF SEQUENCES: 24

   (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Amgen Inc., U.S. Patent Operations/NAO
        (B) STREET: 1840 Dehavilland Drive
        (C) CITY: Thousand Oaks
        (D) STATE: California
        (E) COUNTRY: USA
        (F) ZIP: 91320-1789

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

   (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 774 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CTGCAGGCTC AGAGGCACAC AGGAGTTTCT GGGCTCACCC TGCCCCCTTC CAACCCCTCA      60

GTTCCCATCC TCCAGCAGCT GTTTGTGTGC TGCCTCTGAA GTCCACACTG AACAAACTTC     120

AGCCTACTCA TGTCCCTAAA ATGGGCAAAC ATTGCAAGCA GCAAACAGCA AACACACAGC     180

CCTCCCTGCC TGCTGACCTT GGAGCTGGGG CAGAGGTCAG AGACCTCTCT GGGCCCATGC     240

CACCTCCAAC ATCCACTCGA CCCCTTGGAA TTTCGGTGGA GAGGAGCAGA GGTTGTCCTG     300

GCGTGGTTTA GGTAGTGTGA GAGGGTCCGG GTTCAAAACC ACTTGCTGGG TGGGGAGTCG     360
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TCAGTAAGTG GCTATGCCCC GACCCCGAAG CCTGTTTCCC CATCTGTACA ATGGAAATGA   420

TAAAGACGCC CATCTGATAG GGTTTTTGTG GCAAATAAAC ATTTGGTTTT TTTGTTTTGT   480

TTTGTTTTGT TTTTTGAGAT GGAGGTTTGC TCTGTCGCCC AGGCTGGAGT GCAGTGACAC   540

AATCTCATCT CACCACAACC TTCCCCTGCC TCAGCCTCCC AAGTAGCTGG GATTACAAGC   600

ATGTGCCACC ACACCTGGCT AATTTTCTAT TTTTAGTAGA GACGGGTTTC TCCATGTTGG   660

TCAGCCTCAG CCTCCCAAGT AACTGGGATT ACAGGCCTGT GCCACCACAC CCGGCTAATT   720

TTTTCTATTT TTGACAGGGA CGGGGTTTCA CCATGTTGGT CAGGCTGGTC TAGA   774

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 22 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTCTAGAAAG CTTAATTCAG TC   22

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 28 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCCCCGCGGG GAAGAGCGCA GAGCTCGG   28

(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 22 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTCTAGAAAG CTTAATTCAG TC   22

(2) INFORMATION FOR SEQ ID NO:5:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 31 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGGATCTCG AGGTACCCGG GGATCATAAT C   31

(2) INFORMATION FOR SEQ ID NO:6:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 30 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGTCGACMW CSVTGCMCCH RYMYSMYCYA   30

(2) INFORMATION FOR SEQ ID NO:7:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 29 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGACTAGTCC AGAGCACACA AGCTTCTAG   29

(2) INFORMATION FOR SEQ ID NO:8:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 39 base pairs
     (B) TYPE: nucleic acid

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```
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATAAGAATGC GGCCGCTAAA CTATTGCATC TGGCAACCC                          39

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCTCTAGAA AGAGCTGGGA C                                             21

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCCGTGTTC CATTTATGAG C                                             21

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CAATCTACAA TTCACAGA                                                 18
```

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(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTAAGTTATT GCCATAGG   18

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 29 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AACAAAGCTT CTACAATTCA CAGATAGGA   29

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AACAAGATCT TAAGTTATTG CCATAGG   27

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA